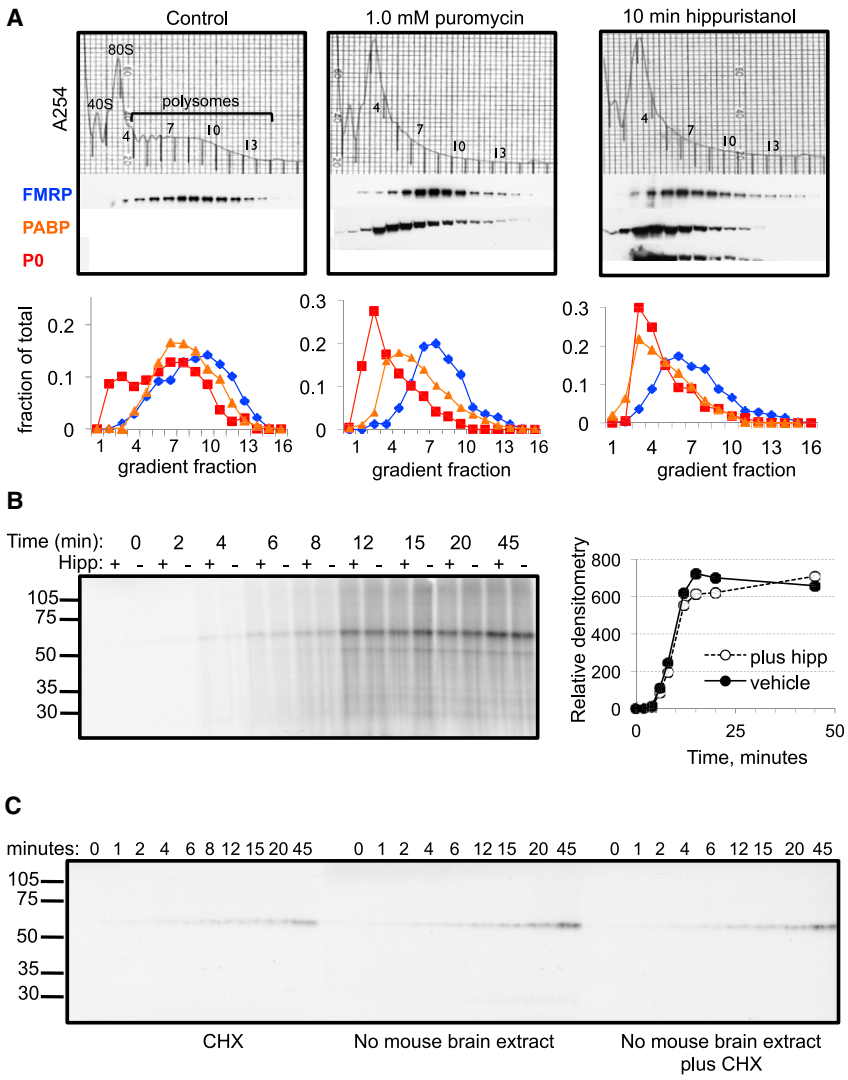

Figure S3. Confirmation that FMRP Is Present in Heavy Sucrose Fractions after Treatment with Translation Inhibitors and Development of an In Vitro Translation System Programmed with Endogenous Brain Polyribosomes, Related to Figure 3

(A) FMRP appears to associate with translationally stalled polyribosomes in neuroblastoma cells. Comparison of A_{254} traces of RNA distribution and Western blots of FMRP (blue diamonds), PABP (orange triangles) and ribosomal protein P0 (red squares) in 20%–50% w/w sucrose gradients from Neuro-2A cells treated with increasing doses of puromycin for 1 hr (middle panel) or 1 uM hippuristanol for 0-10 min (right panel) to induce ribosome run-off, quantified by densitometry/Versadoc imaging and plotted as a fraction of the total in lower panels. FMRP, rpP0 and PABP shifted in a dose- and time-dependent manner; however, only maximal run-off is shown.

(B) Natural elongation in IVT_{EBP} system is accompanied by ^{35}S-methionine incorporation into nascent proteins in a time-dependent and brain polyribosome-dependent manner. Overview: A_{254} traces of total RNA distribution from a time course of puromycin-induced run-off in the brain-programmed in vitro translation (IVT_{EBP}) system was assessed. At time zero the 40S, 60S, 80S and polyribosome-associated fractions were clearly visible on 20%–50% w/w sucrose gradients, and in the absence of added mouse brain extract no polyribosomes was visible (data not shown). During elongation at 30°C ribosome "run-off" (loss of A_{254} signal from polyribosome fractions and increase in 80S monosome fractions) was evident within 2.5 min and was complete at 15 min (no further run-off was observed at 20 min or 45 min when the curves were overlaid). A 10-fold excess of puromycin (72 ug/ml) resulted in no greater run-off than 7.2 ug/ml as evidenced by the A_{254} trace of rRNA distribution. To assess whether RNase activity in the lysate contributed to loss of polyribosomes during incubation at 30°C, reactions were incubated up to 45 min in the presence of CHX without loss of polyribosomes, demonstrating lack of measurable RNase activity in this system. Finally, when the IVT_{EBP} system was allowed to elongate in the absence of puromycin ("natural run-off"). significant ribosome run-off (relative to CHX profiles) was evident. Addition of hippuristanol (an inhibitor of initiation) during natural elongation demonstrated no additional ribosome run-off, suggesting that initiating ribosomes do not contribute to polyribosome size under these conditions. (B) Addition of ^{35}S-methionine to the IVT_{EBP} system during natural run-off demonstrates labeling of new proteins over a wide range of molecular weights in a time-dependent manner, labeled in minutes of incubation at 30°C. Addition of 1.3 uM hippuristanol to parallel reactions (labeled + or -) demonstrates that proteins are synthesized with little contribution from new initiation. (B; right panel): Quantitation of ^{35}S-methionine incorporation into new proteins by densitometry and analysis (Quantity One software) of a group of proteins ranging from approximately 60-100 kDa (above the non-protein synthesis dependent band, see (C)), subtracting the CHX control signal (in (C), demonstrated that new protein synthesis plateaus at 15 min as

suggested by A_{254} traces (data not shown). The 4 min lag in initial labeling may be due to threshold for detection by imaging or time needed for the reaction to warm to 30°C.

(C) As controls, parallel reactions were treated with CHX (left), demonstrating protein labeling in the IVT_{EBP} system is due to new protein synthesis with the exception of a band at approx. 60 kDa whose labeling was not inhibited by protein synthesis inhibitors nor was it dependent on added mouse brain extract. We conclude this band was likely due to a post-translation modification of an existing protein in the rabbit reticulocyte lysate. Labeling in this system was also completely dependent on added mouse brain polyribosomes (middle and right panels).